Whole-exome sequencing of a meningeal melanocytic tumour reveals activating CYSLTR2 and EIF1AX hotspot mutations and similarities to uveal melanoma

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Brain Tumor Pathology

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Response to Reviewers:	Response To Peer Reviews We would like to thank the editorial team and peer reviewers for taking the time to consider our study. We agree with all the comments and suggestions made and have acted on all of them. We greatly appreciate this feedback and are confident that this has improved our manuscript. Comments for Author: Reviewer #1: The authors reported a case of meningeal melanocytic tumor (MMT), associated with a nuchal port-wine stain, with whole exome sequencing analysis to find similarity of gene mutational rate and spectrum of the MMT to those of uveal melanoma (UM). Additionally, they identified several candidate driver mutations of the

MMT.

General comment.
Although genetic similarity between MMT and UM was previously reported and, unfortunately, it remained undetermined whether the port-wine stain was genetically related to the MMT, newly identified candidate driver mutations of the present case would be of interest to the reader.

Respons:
We thank the reviewer for their excellent and accurate summary of our study. We appreciate that the molecular similarity between MMT and UM has been reported based on specific point mutations. Herein, and for the first time, we confirm these findings across the entire exome. We further identify two recurrent mutations in EIF1AX and CYSLTR2 which likely represent driver mutations and we present the data for the remaining 17 somatic protein-coding alterations detected exome-wide. Although, due to the limited available data in this rare disease, we cannot comment on the frequency or functional significance of the other somatic alterations, our hope is that our publically-available data (for both tumour and germline exome sequencing) could form part of larger future analyses in this area.

The reviewer correctly pointed out that the molecular relationship of the MMT to the port-wine stain remained undetermined in this study. In view of the difference in timing of appearance between these lesions (congenital port-wine stain versus MMT presenting in the eighth decade of life) as well as the entirely different cell of origins in these anatomically distinct locations, our biological hypothesis is that these lesions are unlikely to be molecular related. However, due to the very small number of mutations in the MMT and lack of DNA remaining in the port-wine stain, this would require a repeat-biopsy of the port-wine stain to conclusively prove which we do-not feel would be in the patients' best clinical interests. For clarity, we have added a comment that there were no overlapping mutations between these lesions across the sequenced chromosomal regions, and that further research will be required to determine if there is any plausible molecular relationship.

Specific comment.
Some of the reference number in the main text seems to be incorrect.

Respons: The reviewer correctly pointed this out and a correction in the reference numbering in the text was made.

Reviewer #2: This study examined a case of meningeal melanocytic tumor of intermediate malignancy and an associated port-wine stain by whole-exome and targeted sequencing, respectively. The authors confirmed the presence of previously reported mutations in CYSLTR2 and EIF1AX and a relatively small number of additional novel mutations in the MMT. The mutational profile was different from cutaneous melanoma and similar to uveal melanoma, as previously reported. The associated port-wine stain did not harbor mutations in GNAQ, GNA11, or other vascular tumor associated genes in the targeted sequencing.

The study is interesting and, although mostly confirmatory, provides (or potentially could provide) some novel data. I have following comments:

1. Except for the known mutations of uveal melanoma or MMT (CYSLTR2 and EIF1AZ), the significance of mutations identified is quite unclear. Although the authors showed that at least 3 of them are recurrent based on the data in other cancer types, the recurrence itself does not prove its significance. The authors may want to use several software-based estimation (eg, PolyPhen etc.) as to how detrimental each mutation could be. In this regard, the title " new candidate driver mutations" appears an overstatement, as there is no proof of driving roles of these genes in the paper.

2. Because the MMT in this case lacked mutations in GNAQ/GNA11 or vascular-tumor related gene, it is not so interesting to test these mutations in the associated

	port-wine stain. It is more reasonable to know whether any of the mutations identified in the MMT exists also in the vascular tumor. Respons: We would like to thank the reviewer for their thorough summary and for identifying all the salient points in this study together with the key limitations. We thank the reviewer for their accurate comment on the three other point mutations we described in CENPV, TSPAN18 and TNIP3. We were surprised to find that (of such few somatic alterations in the MMT) variants at these exact nucleotide positions had been previously reported in other cancers. Although the probability of this happening by chance is scarce, we do agree that recurrence in itself does not fully prove significance. Running these positions through both PolyPhen-2 and SIFT does indicate that these mutations are probably damaging (PolyPhen-2 score 1.00, 0.927 and 1.00 and SIFT score: 0.09, 0.05 and 0 for each mutation respectively). We do agree that this not sufficient evidence to present these as potential driver mutations and have amended this statement significantly, highlighting that their biological significance is as yet undetermined and will clearly require further study. We accept the comment on the manuscript title which we have amended accordingly, we believe this accurately summarises our key findings. Our hope is that this publically available (tumour and normal) sequencing data could form part of larger future analyses which could better determine the biological significance of these other mutations. As the reviewer pointed out, it is important that we draw attention to our findings of mutations in EIF1AX and CYSLTR2 at these nucleotide positions. These mutations had a high variant allele frequency of 0.86 and 0.48, respectively, and have been previously reported in MMTs as described. The reviewer highlights an excellent point regarding the possible relation of the variants detected in the MMT to the port-wine stain. The panel sequencing of the port-wine stain (which preceded the whole-exome sequencing of the MMT) was focussed around key melanoma-driver and vascular-associated genes. The genes selected for this panel were based on our biological hypotheses at the time and in retrospect, given the evidence gleaned from the whole-exome sequencing and recent studies showing the absence of melanoma-drivers in these tumours, we would have been keen to interrogate the newly-identified MMT-associated alterations in the vascular lesion. Due to the very limited number of mutations and lack of DNA in the port-wine stain a repeat-biopsy of the port-wine stain would be the only way of conclusively answering this question. This is technically challenging due to the very low yield of neoplastic cells from any such biopsy and we do not feel this would be in the patients' clinical best interests. Biologically, in view of the difference in timing of appearance between these lesions (congenital port-wine stain versus MMT presented in the eighth decade) as well as the entirely different cell of origins in these anatomically distinct locations, our hypothesis is that these lesions are unlikely to be molecular related, however this will require further study. For clarity, we have added a comment that there were no overlapping mutations between these lesions across the sequenced chromosomal regions, and that further research will be required to determine if there is any plausible molecular relationship.

Whole exome sequencing of a meningeal melanocytic tumour reveals activating *CYSLTR2* and *EIF1AX* hotspot mutations and similarities to uveal melanoma ~~reveals new candidate driver mutations~~

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Keywords meningeal melanocytic tumour; melanocytoma; port-wine stain; spinal cord neoplasm; whole exome sequencing; *CYSLTR2*; *EIF1AX*

To the Editor,

Melanocytic tumours originating in the leptomeninges range from low-grade melanocytomas to malignant melanomas. These tumours, referred to as meningeal melanocytic tumours (MMT), occur at a median age of 45 years and have a predilection for the cervical/thoracic spine and the posterior cranial fossa [4]. MMTs are sometimes associated with melanocytic nevi of the skin, however, the association with vascular lesions is not well documented [4]. MMTs share certain mutations with uveal melanoma (UM), including hotspot mutations in *GNAQ*/*GNA11* and lack *BRAF* mutations frequently present in cutaneous melanoma (CM) [~~8~~4]. Herein we report the first whole exome sequencing analysis of a cervical intradural MMT, associated with a port-wine stain.

This previously healthy 85-year-old male presented with progressive cervical pain and mild dysesthesia in both hands. Physical examination was unremarkable. Other than a large congenital nuchal port-wine stain, no other cutaneous lesions were identified (**Figure 1a**). Magnetic resonance (MR) imaging showed a homogenous enhancing intradural extramedullary tumour extending from C2 to C4 compressing the spinal cord (**Figure 1b,c**). Cervical laminectomy and opening of the dura revealed a dark-colored extramedullary lesion (**Figure 1d**) firmly adherent to the pia. As a result, subtotal resection was performed and histology revealed a melanocytic tumour of intermediate-grade malignancy (**Figure 1e**). DNA methylation profiling confirmed its primary origin in the central nervous system (**ESM_1**). The patient received no additional therapy. Follow-up at two years showed no neurological deficits or growth of the residual tumour. Formalin-fixed and paraffin embedded material from the MMT was available for whole exome sequencing. A biopsy of the port-wine stain underwent cancer-related and selected vascular-associated gene panel sequencing (**ESM_1**).

Whole exome sequencing of the MMT (alongside matched germline DNA) revealed only 27 somatic mutations in protein-coding regions of the genome, of which 19 were protein-altering and 8 were silent (**Figure 2a-b, ESM_2**). This low number of mutations is in stark contrast to CM and may reflect the lack of UV-exposure (48% of all nucleotide changes were cytidine to thymidine (C>T) transitions) (**Figure 2c**). Comparing both the mutational rate and spectrum of this MMT to those of 80 primary UM [1] revealed a median correlation coefficient of 0.973, which is significantly higher than the corresponding median correlation across 64 primary CM ($p=1.725x10^{-11}$). This further supports genetic similarities between MMTs and UM (although the possibility FFPE deamination should be considered) (**Figure 2c, ESM_3**).

In keeping with a recent report by van de Nes *et al* [10], a hotspot mutation in *CYSLTR2* (c.386T>A, p.L129Q) was detected in the MMT. In that study, *CYSLTR2* mutations were mutually exclusive from mutations in *GNAQ/GNA11,* suggesting an independent driver in MMTs, as was the case in our patient. We also found a mutation in *EIF1AX* (c.7A>G, p.K3E, previously reported in UM [5]), with mutations in this gene occurring in conjunction with *CYSLTR2* in MMTs [10].

Of the remaining 17 protein-altering mutations, ~~T~~three other exact nucleotide positions were found to be somatically mutated in the cBio catalogue of cancer mutations [1]; in *CENPV* (c.701C>A, p.A234D), *TNIP3* (c.712C>T, p.R238C) and *TSPAN18* (c.158C>T, p.T53M), as reported in carcinomas of the breast [7], skin (squamous cell) [2] and stomach [6], respectively. These three mutations were predicted to be deleterious in both PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/bgi.shtml)

and SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), however their recurrence and biological significance is as yet undetermined. Sequencing reads from all variants were visually inspected on the Integrative Genomics Viewer (IGV) Software [8] and mutations in *EIF1AX* and *CYSLTR2* were validated by capillary sequencing (**ESM_1**).

There is only one report of a thoracic MMT associated with a sacral port-wine stain [3]. The anatomical proximity of the port-wine stain to the MMT in this patient is reminiscent of phacomatosis pigmentovascularis (port-wine stain associated with pigmented lesions of the skin and eye), characterised by somatic *GNAQ* or *GNA11* mutations as in MMTs [9]. However, no mutations were detected in *GNAQ/GNA11* or in other vascular-associated genes within our panel in the port-wine stain (**ESM_4**). We found no shared mutations between the MMT and the port-wine stain across the sequenced regions and tThe biological relationship of the MMT to the port-wine stain in this case therefore remains unclear.

~~Herein we provide further evidence that MMT is distinct from CM and demonstrate a similar mutational rate and spectrum to UM. We also identify new candidate driver mutations in MMTs which require further validation.~~

Herein we provide further evidence that MMT is distinct from CM and for the first time, demonstrate molecular similarities between MMT and UM across the entire exome. We also identify recurrent mutations in *CYSLTR2* and *EIF1AX* which likely represent driver mutations.

Abbreviations

UM: uveal melanoma; CM: cutaneous melanoma

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We would like to thank the patient for allowing us to undertake these analyses and to share these data. We thank Dr. J. Koten for critically revising the manuscript. We are grateful to Marcel Jeunink for assistance with the molecular analyses.

Availability of data and materials

Data accession through the European Genome-phenome Archive (dataset accession ID EGAD00001003750).

Study accession IDs: EGAN00001493783 - tumour of index patient.

EGAN00001493784 - germline of index patient.

Conflicts of Interest

The authors report no conflicts of interest.

Ethical approval and consent to participate

Ethical approval was sought from the Commisie Mensgebonden Onderzoek, Nijmegen, Netherlands (CMO, ref.nr/Dossiernummer: 2016-2863). Written informed consent was obtained from the patient for publication of this case report and its accompanying images. A copy of the written consent is available for review by the editor.

Author contributions

HK, MG and ML designed the study, made substantial contributions to the acquisition and interpretation of data and wrote the manuscript. RR and DJA made substantial contributions to the acquisition and interpretation of molecular data, and were major contributors in writing the manuscript. MR, RB, CP and WB contributed to the acquisition and interpretation of molecular data and WB and HK reviewed the histology. All authors were involved in drafting the manuscript or in its critical revisions, and participated sufficiently to take public responsibility for appropriate portions of the content. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Port-wine stain in the neck; 6 months postoperative (**A**), MR imaging, axial T1 weighted with contrast, level C3-4; homogenous enhancing intradural extramedullary tumour (arrow) with spinal cord compression (**B**), MR imaging, sagittal T1 with contrast; tumour extending from C2 to C4 (**C**), laminectomy revealed a dark-colored solid dural lesion (40mm in long axis). (**D**), Histology; pigmented spindle cell tumour with mitotic activity (arrow) (scale bar 50µm) (**E**).

Figure 2. Mutational spectrum of the index patients' tumour, showing all variants. Only 13/27 (48%) of all nucleotide changes were cytidine to thymidine (C>T) transitions (**A**). Non-synonymous mutations identified in the MMT (**B**). Comparison of mutational rate across this MMT, primary UM (n=80) and primary CM (primary tumours only indicated) (n=64). Only

non-synonymous variants are shown (log_{10} scale). Data accession from the cBio catalogue of cancer mutations [1] (**C**).

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Figure 1

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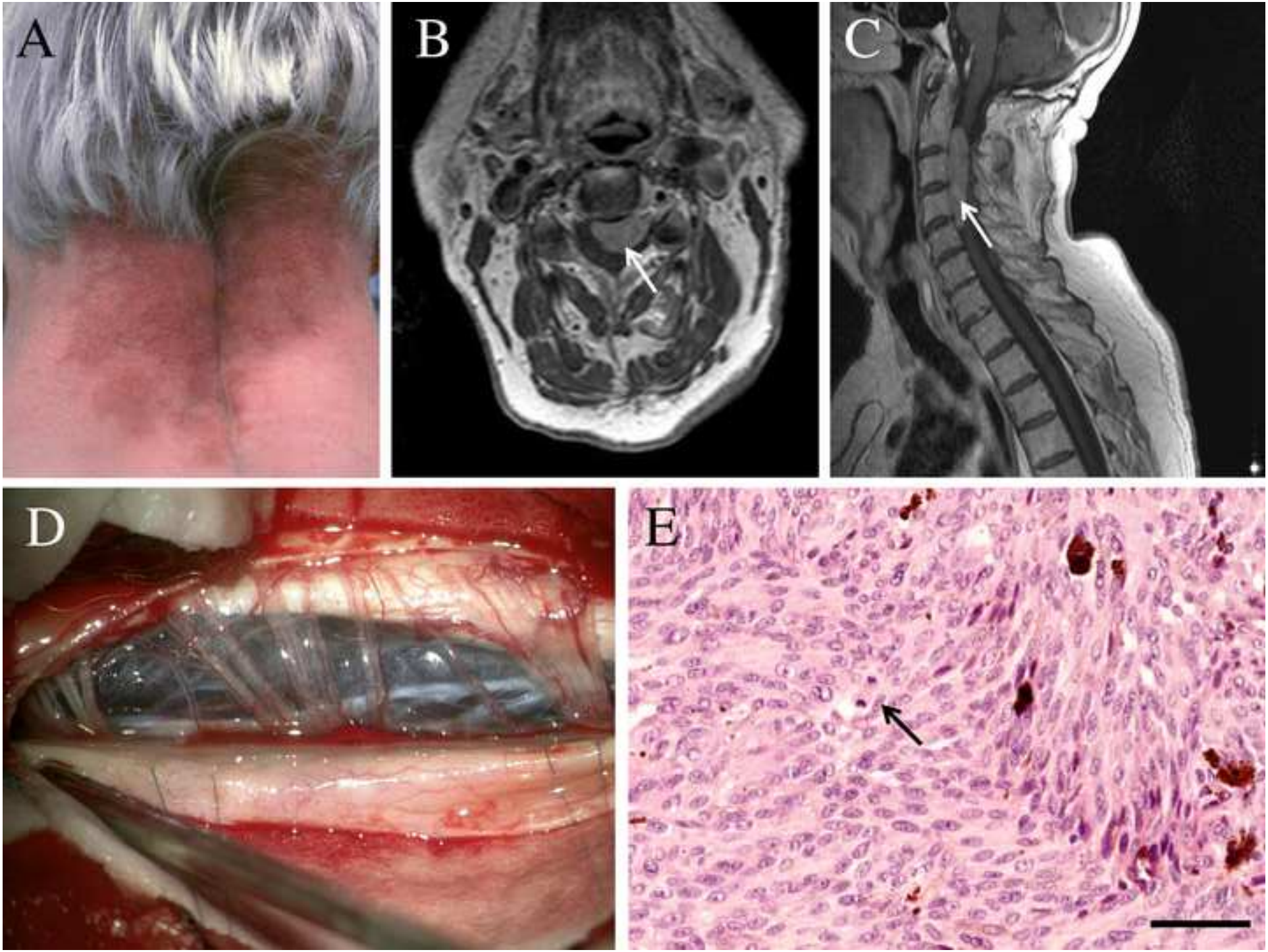

Figure 2

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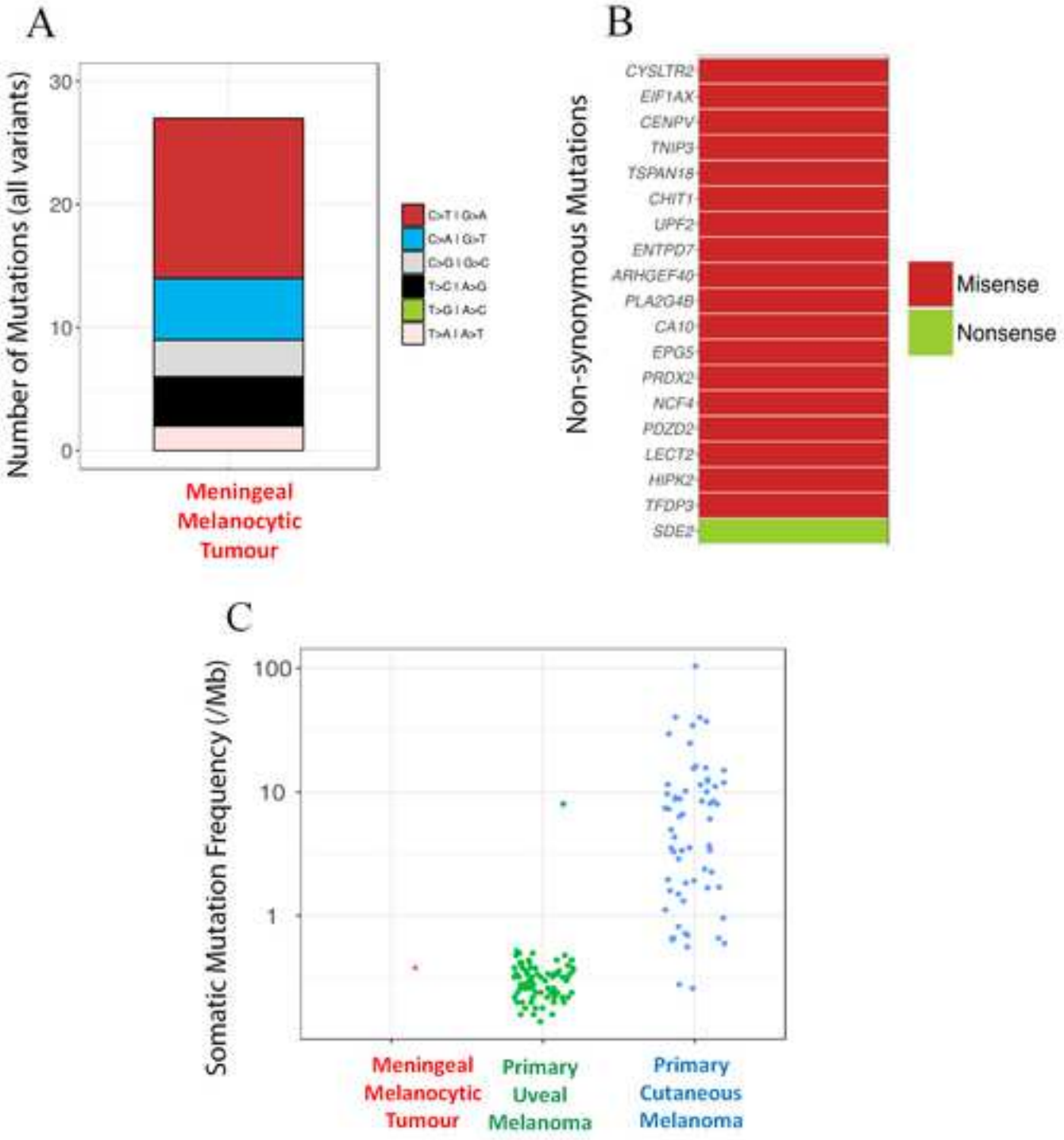

ESM_1

ESM_3

ESM_4